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14. ABSTRACT

p63 is a member of the p53 gene family, and shows structural and functional similarities to the p53 tumor suppressor. While p53's role in breast carcinogenesis is well established, p63's involvement in this disease remains unclear. It has been shown that p63 is expressed in the myoepithelial cells of the breast, and that p63 is essential for mammary development.
The main goal of this project is to investigate the potential role of p63 in breast cancer. Despite the homology to p53, p63's functions and mechanisms cannot necessarily be extrapolated from p53 paradigms. To understand the mechanisms of transcriptional regulation by p63, we completed an analysis of *in vivo* p63 DNA-binding sites across the entire human genome. We provide evidence for the biological relevance of the binding sites identified, including motif discovery and evolutionary conservation. We also used RNAi strategies to analyze the consequences of p63 deficiency. By combining data from expression profiling of p63-depleted cells with the *in vivo* binding data, we identify a subset of genes that are directly regulated by p63. These include genes in cell proliferation, apoptosis, and various signaling pathways. Together, our data provide a platform for studying p63 in cancer and developmental pathways.

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Introduction:

p63 is a member of the p53 gene family, and shows structural and functional similarities to the p53 tumor suppressor [1,2]. While p53's role in breast carcinogenesis is well established, p63's involvement in this disease remains unclear [3-6]. It has been shown that p63 is expressed in the myoepithelial cells of the breast, and that p63 is essential for mammary development.

The main goal of this project is to investigate the potential role of p63 in breast cancer. To this end, a key step is the identification of p63 transcriptional targets *in vivo*, and a comparison to other members of the p53 family of transcription factors. These studies are aimed at elucidating the effectors and mediators of p63's function in development and tumorigenesis, as well as provide insights into interactions with the other family members, p53 and p73, both of which have been implicated in breast and other cancers.

Body: (note: text is adapted from Yang et al., submitted – see Reportable Outcomes)

During this project period, we made additional progress on aims described in Task 2 (Statement of Work), and addressed questions raised in Task 1 regarding the effects of p63 depletion. Of note, we have continued to use ME180 cells as the primary experimental system for our studies to date, in place of the MCF10A cells described in the original proposal. As explained in the previous Annual Report, ME180 cells were used in the large-scale chromatin immunoprecipitations (ChIP) because they express high levels of p63 protein, and were optimized for the ChIP experiments. Given p63's essential and broad role in epithelial development, we anticipate that there will be considerable overlap between transcriptional targets identified in a cervical epithelial (ME180) vs. mammary epithelial (MCF10A) cell line. It should also be possible to carry out downstream experiments in mammary-specific systems. As a whole, these approaches should still adhere to the overall goal of understanding p63 function.

Task 1: Analyze the effect of p63 deficiency and overexpression on mammary epithelial cells

Since the whole genome analysis of p63 targets has been performed in ME180 cells (see Task 2 below), we felt it was important to analyze the consequences of p63 depletion in the same cells, in order to correlate our *in vivo* DNA binding data with transcriptional effects mediated by p63. ME180 cells were transduced with a small hairpin RNA (shRNA) specific for the p63 oligomerization domain, resulting in a 5-10 fold reduction in p63 levels. RNAs from three independent replicates of p63-depleted and control cells were hybridized to Affymetrix GeneChip arrays containing probes sets for ~20,000 unique human genes. We determined genes that were differentially expressed between control and $p63^{shRNA}$ cells, using false discovery rate (FDR) cut-offs. At FDR ≤ 10%, we found 1035 differentially expressed genes, with 458 (44%) being down-regulated and 577 (56%) being up-regulated upon depletion of p63.

The relationship between p63 binding and p63-dependent transcriptional changes was investigated by ranking genes based on their respective FDRs for differential expression [7-8] and comparing the average differential expression rank for p63-bound genes to an equal number of randomly sampled genes. Genes bound by p63 ranked better than any of the 10,000 random groups, indicating a significant ($P<0.0001$) correlation between p63 binding and p63-dependent changes in mRNA expression. The same result was observed with a stepwise approach, in that a larger than expected number of p63-bound genes are found towards the more differentially expressed end. The only exceptions were genes at the very top (first bin) of the list, which may

reflect the activation of stress pathways or other responses to the morphological changes and apoptosis seen in $p63^{shRNA}$ cells.

Although there is a significant relationship between p63 binding and p63-dependent transcriptional activity, this relationship is complex. First, only15-17% (depending on FDR cut-off) of genes that display p63-dependent changes in RNA levels show p63 binding in the vicinity of the gene. This observation might reflect indirect effects of p63 depletion, and in this regard, p63 is a key regulator of epithelial cells [9-10] that may influence expression changes beyond its direct targets, and p63-depleted ME 180 cells undergo apoptosis shortly after the time when RNA was isolated. It is also possible that some of these p63-regulated genes might be directly affected by p63 bound at relatively large distances from the gene as defined here.

Conversely, although our results identify many p63 sites that directly affect transcriptional activity, the majority of the 2247 genes bound by p63 do not exhibit changes in mRNA expression upon p63-depletion in ME180 cells. Even at an FDR of 20%, less than a quarter of p63-bound genes show transcriptional effects in $p63^{shRNA}$ versus control cells. It is likely that many more p63 target sites are transcriptionally competent but not detected due to limitations of the analysis and the fact that transcriptional effects were assayed under only a single physiological condition. Interestingly, binding sites for the subset of p63 targets that are associated with p63-responsive transcriptional effects are significantly more conserved through evolution than typical p63 sites, providing additional evidence that these sites are biologically important.

Lastly, we noted a significant number of genes that are bound by p63, and whose expression is upregulated in p63-depleted cells. We also showed that ΔNp63 protein is the only detectable isoform in ME180 cells. Together, our data strongly suggest that ΔNp63 isoforms, which lack the canonical p53 transactivation domain, are activators of gene expression *in vivo*.

Task 2: Identify novel transcriptional targets of p63 and p53

We have now completed identification and analysis of p63 DNA binding sites using tiled, high-density microarrays covering the entire human genome. These studies fulfill Task 2 (Statement of Work), albeit with ME180 cells. We attempted similar experiments to identify p53 targets, but our data and other evidence suggest that the p53 protein in ME180 cells, although detectable and wildtype for sequence, is not functional for DNA binding. Our efforts are therefore focused on p63, and summarized below:

(a) identification and characterization of p63 binding sites

We used the ME180 cervical carcinoma cell line, which expresses abundant p63, to create a high-resolution map of p63-DNA interactions *in vivo*. DNA from three independent ChIP experiments with the 4A4 anti-p63 antibody was hybridized to tiled, oligonucleotide microarrays that interrogate the non-repetitive sequences of the human genome at roughly 35 basepair (bp) resolution. Approximately 5800 and 3700 binding sites were identified for (-) and (+) Actinomycin D (Act D) conditions, respectively. We validated the array-based data by direct analysis of a subset of p63 targets using quantitative real-time PCR (qPCR). This analysis yielded high confidence for sites above a p-value cut-off of 10E-5, with an estimated false positive rate of ~9%. Lower false positive rates were obtained at using higher cut-offs, but significantly raised the false negative rates such that many bona fide p63 targets would be missed.

We observed a strong overlap between binding targets in (-) and (+) Act D samples, suggesting that while Act D treatment results in decreased p63 protein levels and genome occupancy, it does not alter p63 binding specificity in ME180 cells. Moreover, the majority of p63 binding sites reside in non-canonical locations – that is, although we see enrichment at promoter and upstream regions of genes, a significantly large number of sites are found within intronic regions. Specifically, there is a strong preference for intron 1. We further note that despite a general association with 5'ends of genes, p63 binding sites are frequently located at large distances (> 10 kb) from transcriptional start sites. These observations suggest long-range regulation of gene expression, and underscore the value of unbiased and comprehensive (i.e. whole genome) approaches for identification of transcription factor binding sites. Finally, while a significant percentage (~54%) of p63 binding sites are found in the vicinity (defined as within 5 kb upstream to 1kb downstream) of known genes, many sites are not associated with gene annotations in existing databases. We anticipate that future advances in annotation of the human genome – including novel transcripts, non-coding and micro RNAs – will improve the association of p63 sites with relevant targets.

(b) de novo identification of p63 binding motif

We used *de novo* motif discovery algorithms MEME and AlignACE to define sequence elements shared by the in vivo p63 binding sites we identified. This analysis revealed a dyad-symmetric motif comprised of two direct repeats, with similarity to the consensus motif for p53 binding. We also showed that motif quality was correlated with binding strength in vivo. Importantly, however, only 8% of the best p63 motifs and only 1-2% of typical p63 motifs (i.e. found in majority of p63-bound sites) are actually bound *in vivo*. Conversely, many p63-bound sites show motif scores similar to those found randomly in the genome. Together, these findings show that despite a significant correlation, motif score *per se* is a poor predictor of binding *in vivo*. Our data also highlight the notion that binding *in vivo* is highly selective, and may require additional factors and/or chromatin accessibility.

(c) biological relevance of p63 binding sites

The observation that p63 interacts with a broad array of genomic loci, including many in non-canonical locations, raises the question of whether they are all biologically relevant. We provide several lines of evidence to support this notion. First, p63 binding sites show strong evolutionary conservation in numerous species, as compared randomly selected regions. Second, p63-bound regions are also enriched for sequence motifs for other transcription factors. Specifically, we noted a statistically meaningful enrichment for factors such as NF-AT, c-Ets-1, and STAT5, among others. Finally, we provide evidence for direct transcriptional targets of p63 – using RNAi strategies, we were able to correlate p63 binding with p63-dependent effects on mRNA expression. Together, these support the validity and functional relevance of p63 binding sites identified in our study.

(d) Functional categories for p63 targets

The whole-genome analysis of p63 binding sites has provided a comprehensive view of target genes. The p63 targets uncovered in our study include genes previously linked to p63, such as CDKN1A/p21, BBC3/PUMA [11], and DST/BPAG [12],. As defined by Gene Ontology [13], the subset of "direct" targets (i.e. bound by p63 and show transcriptional changes upon p63 depletion) is enriched (FDR<0.05) with genes involved in cell cycle, cell death, and cell

proliferation. This observation highlight the functional connection between p63 and p53 to p53 in growth arrest and apoptosis, and it reveals novel targets that may be regulated by both transcription factors in these processes.

A similar analysis on the entire set of p63 binding targets identifies many p63-bound genes involved in protein kinase activity (P=2.10E-8). In particular, p63 appears to be involved in several canonical signaling and developmental processes. At least 24 p63 targets are associated with Notch signaling, including the JAG1 gene [14], and p63 also binds multiple components of the Wnt and TGF signaling cascades. The Notch, Wnt, and TGF pathways are implicated in epithelial morphogenesis and stem cell biology [15-18], and effects on these pathways may contribute to the molecular basis underlying the phenotypes of p63 deficiency.

Task 3. Test functional interactions between p63 and p53 transcriptional regulation

The objective here was to identify and compare binding sites for the family members, and determine whether these homologs affected each other's transcriptional regulation and function. We have experienced difficulties in identifying targets for p53, as discussed above. However, during the course of our work on p63, a genome-wide analysis of p53 binding sites in HCT-116 cells was reported [19]. Using these data, we were able to compare *in vivo* binding sites and behavior for both factors. Of note, the p53 experiments involved a SAGE-based (rather than array) strategy – interestingly, they identified far fewer high-confidence sites (327) than we did for p63, or than might be expected based on previous p53 ChIP-chip experiments on limited portions of the genome [20]. In any case, we observed a striking overlap between binding sites for p53 and p63. 62 (out of 327) targets were shared between the two factors (P ~2.4 x 10^{-70}). Nevertheless, the remaining p53 sites show very poor binding enrichment scores for p63 in our experiments, indicating these differences reflect distinct binding preferences of the two proteins *in vivo*. However, these conclusions are somewhat limited by the fact that these experiments were not done using the same cell types or techniques.

To better address the notion of functional interactions among p53 family members, we have begun experiments to identify binding targets for p73. These are being done in ME180 cells, so that we can make the most use of the vast p63 data obtained to date. p73 shows even stronger homology with p63 than either does with p53. A comparison of *in vivo* targets should allow us to ask whether these factors bind to similar sequences, what the basis of their recruitment to target sites is, and whether they can compete or cooperate with one another in DNA binding and gene regulation.

Key Research Accomplishments:

- Identified ~5800 p63 DNA binding sites across entire human genome (ME180 cells)
- Discovery of an *in vivo* DNA motif for p63 binding; showed motif score is generally correlated with binding strength. However, a strong motif is neither sufficient nor necessary for p63 binding *in vivo*.
- Showed that Actinomycin D treatment decreases p63 protein levels and site occupancy, but does not affect binding specificity in ME180 cells
- Showed that p63 binding sites are preferentially located in promoter regions and intron 1 of associated genes, and also at large distances away from annotated genes.

- Showed that p63 binding sites are more evolutionarily conserved than random expectation
- Showed that p63 binding sites contain additional motifs for other transcription factors
- Showed that p63 binding sites overlap with p53 targets (Wei et al., 2006), but that these two related factors likely have distinct targets as well.
- Used lentiviral RNAi strategies to deplete p63 expression in ME180 cells. Performed mRNA expression profiling of $p63^{shRNA}$ cells, correlated RNAi data with *in vivo* binding data to determine subset of "direct" transcriptional targets for p63.
- Established that transcriptional activation is a common and physiological mechanism of action for ΔNp63 isoforms that lack a canonical transactivation domain
- Showed that p63 targets are enriched for genes in cell cycle, proliferation, death, cell adhesion, and various signaling pathways

Reportable Outcomes:

Yang A*, Zhu Z*, Kapranov P, McKeon F, Church G, Gingeras GR, Struhl K. (2006). Identification, selectivity, and functional analysis of p63 target sites across the human genome. Submitted. (* denotes equal authorship)

Yang A*, Zhu Z*, Kampa, D., Kapranov P, McKeon F, Church G, Gingeras GR, Struhl K. Transcription and Functional Activities of the p53 Gene Family. Abstract, poster presentation at the 2005 Era of Hope Conference, Philadelphia, PA.

Yang A*, Zhu Z*, Kapranov P, McKeon F, Church G, Gingeras GR, Struhl K . Transcriptional Targets and Functional Activities of the p53 Family. Abstract, poster presentation, submitted for the Beyond Genome 2006 Conference, San Franciso, CA.

Conclusions

We have made significant progress on various aims described in the proposal. The genome-wide identification and analysis of p63 targets *in vivo* should greatly facilitate efforts to understand its role in development and cancer. Ongoing experiments to compare these data to the other homologs should offer additional insights on functional interactions among the p53 family.

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